Chapter 5
Phenotyping for Groundnut (*Arachis hypogaea* L.) Improvement

Janila Pasupuleti and S.N. Nigam

Abstract Groundnut (*Arachis hypogaea* L.) is grown world over for oil and food uses. It is a self-pollinated crop with low genetic diversity. The origin of the crop from single hybridization event followed by chromosome doubling as well as crossing barriers of cultivated species with wild species due to ploidy differences rendered the crop with narrow genetic variability. Developing new varieties with increased yield potential and resistance to biotic and abiotic stresses that meet the needs of the growers, processors and consumers is the primary objective of groundnut breeding. In this chapter, we discuss about phenotyping tools used in groundnut improvement programs for various targeted traits. Both field and laboratory tools are described to screen for resistance to diseases caused by fungi, bacteria, virus and nematodes. Phenotyping based on Cumulative Thermal Time (CTT) is used to select for early maturity. Phenotyping for complex traits can be challenging. Either empirical approach that involves measuring the yield under imposed drought stress or salinity conditions or trait based approach using surrogates or a combination of both are used for phenotyping abiotic stresses. Phenotyping for *Aspergillus* contamination needs improvement to derive reliable and reproducible results. Estimation of quality and nutritional parameters generally involves use of destructive and laborious chemical or physical procedures. Near infrared reflectance spectroscopy (NIRS), a robust and non-destructive method is gaining popularity for estimation of oil, protein, carbohydrate and fatty acid contents. Methods for estimating oil, protein, sugar and micronutrient concentrations and fatty acid composition of seeds and haulm quality traits are described.

Keywords Groundnut • Phenotyping • Screening • Yield • Resistance • Disease • Drought • Quality

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5.1 Introduction

5.1.1 Global Importance of Groundnuts

Groundnut, also known as peanut, was grown on nearly 24.07 million ha worldwide with a total production of 37.64 million tons and an average dry pod yield of 1,564 kg ha^{-1} in 2010 (FAOSTAT 2012). China, India, Nigeria, USA, Senegal, Myanmar, Indonesia, Sudan (undivided), Argentina, Ghana and Vietnam are the major groundnut growing countries in descending order and totally account for 84 % of the world groundnut production. Countries in Asia, Africa and South America account for over 97 % of the world groundnut area and about 95 % of the world groundnut production. Production is concentrated in Asia (50 % of the global groundnut area and 64 % of the global groundnut production) and Africa (46 % of the global groundnut area and 28 % of the global groundnut production), where the crop is grown mostly by smallholder farmers under rainfed conditions with limited or no inputs. In the last decade (2000–2010), the global groundnut production increased marginally. The annual increase in production was 0.4 % which was due to both, an annual increase in yield (0.1 %) and area (0.3 %) (Fig. 5.1). The increase in global production of groundnut, contributed by increase in yield is a result of adoption of improved varieties and/or better crop management practices. In India, it is reported that improved varieties alone contributed to 30 % increase in pod yield over two decades (Reddy and Basu 1989).

European Union is the major importer of groundnut oil and groundnuts with and without shells; the value of imports in 2009 was about 254 million USD (FAOSTAT 2012). During the same year, Argentina stood first in groundnut oil exporting

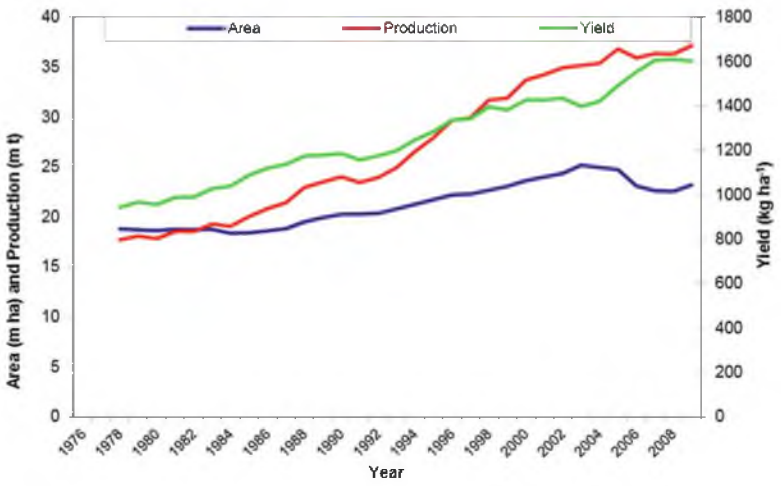

Fig. 5.1 Three-year moving center average for groundnut yield, production and area harvested in world

countries with a value of 84 million USD, while India was the top exporter of shelled groundnut (of value 28 million USD) and China of groundnut with shells (of value 53 million USD) (FAOSTAT 2012).

Groundnut seed can be consumed raw (non-heated), boiled, and roasted or crushed for edible oil. Its haulms are used as animal feed and shells that constitute about 25 % of the total pod mass are used as fuel, as filler in the feed and fertilizer industries and in manufacture of particle boards etc. Globally, over 50 % of the groundnut produced is crushed for extraction of oil for human consumption and industrial uses and slightly less than 40 % is used directly as food, raw or processed as snack (Birthal et al. 2010). The cake obtained after extraction of oil is used in animal feed industry, in making enriched easily digestible food for children and aged persons and as soil amendment. In USA about 75 % of the production is used as food, while in Asia only 35 % is used for food purposes. Groundnut oil is an excellent cooking medium because of its high smoking point (Singh and Diwakar 1993). In USA, Canada, and Australia, groundnut is grown to make peanut butter rather than to extract oil. Groundnut is also used to make confectioneries and its flour to make baked products.

Groundnuts seeds are rich in energy due to high oil (48–50 %) and protein content (25–28 %); they provide 564 K calories of energy from 100 g of kernels (Jambunathan 1991). The seeds contain many health enhancing nutrients, minerals, antioxidants and vitamins and are rich in mono-unsaturated fatty acids. They contain antioxidants like p-coumaric acid and resveratrol and are excellent source of vitamin E and many important B-complex groups of thiamin, pantothenic acid, vitamin B-6, folates and niacin. Groundnut is a dietary source of biologically active polyphenols such as the stilbene trans-resveratrol (Sobolev and Cole 1999), flavonoids (Wang et al. 2008) and isoflavones. Groundnut haulms constitute nutritious fodder for livestock. They contain protein (8–15 %), lipids (1–3 %), minerals (9–17 %), and carbohydrate (38–45 %) at levels higher than cereal fodder. The digestibility of nutrients in groundnut haulm is around 53 % and that of crude protein is 88 % in animals. Haulms release energy up to 2,337 cal kg^{-1} of dry matter.

5.1.2 Taxonomy and Classification

Groundnut (*Arachis hypogaea* L.) is an annual herb belonging to the family Fabaceae (Leguminosae). It is an allotetraploid (2n = 2x = 40) with "A" and "B" genomes. All species, except the cultivated species (*A. hypogaea*) and *A. monticola* in Section *Arachis*, and certain species in Section *Rhizomatosae*, are diploid (2n = 2x = 20). The diploid progentors, *A. duranensis* and *A. ipaensis* contributed "A" and "B" genomes, respectively, to the cultivated groundnut (Kochert et al. 1996). A single hybridization event between the diploid progenitors followed by chromosome doubling about 3,500 years ago lead to origin of cultivated groundnut. The "A" genome set of chromosomes are relatively smaller than the "B" genome. Southern Bolivia and Northern Argentina are thought to be center of origin of this

crop (Gregory et al. 1980; Kochert et al. 1996). The center of diversity of the genus includes Western Brazil, Bolivia, Paraguay and Northern Argentina (Gregory et al. 1980). *A. duranensis* occurs throughout the region, while *A. ipaensis* has only been found in Southern Bolivia. The genetic diversity of the genus is classified into four gene pools (Singh and Simpson 1994): primary genepool consisting of *A. hypogaea* and *A. monticola*, secondary consisting of diploid species from Section *Arachis* that are cross compatible with *A. hypogaea*, tertiary consisting of species of the Section Procumbentes that are weakly cross-compatible with *A. hypogaea* and fourth genepool consisting of the remaining wild *Arachis* species classified into seven other sections.

The cultivated groundnuts are classified into two subspecies, subsp. *fastigiata* Waldron and subsp. *hypogaea* Krap. et Rig. The subsp. *fastigiata* contains four botanical varieties, var. *vulgaris*, var. *fastigiata*, var. *peruviana* and var. *aequatoriana*. The subsp. *hypogaea* contains two varieties, var. *hypogaea* and var. *hirsuta*. Each of these botanical types has different plant, pod and seed characteristics (Krapovickas and Gregory 1994). The *A. hypogaea* subsp. *hypogaea* has alternate pairs of vegetative and reproductive axes on branches (alternate branching) and does not bear flowers on the main axis, inflorescence is simple, generally has two seeds per pod, with moderate seed dormancy, seed coat is generally tan in colour and medium to late maturing. In var. *hypogaea*, cultivars with medium seed size are runner market type and those with large seeds are Virginia market type. In contrast, *A. hypogaea* subsp. *fastigiata* var. *vulgaris* (Spanish market type) has floral axes on main stem, irregular pattern of vegetative and reproductive branches with reproductive branches predominating on branches (sequential branching), inflorescence compound, mostly two seeds per pod and with little or no dormancy. The *A. hypogaea* subsp. *fastigiata* var. *fastigaita* (Valencia market type) has floral axes on main stem, sequential branching, inflorescence usually simple, two or four seeds per pod and little or no seed dormancy.

As a consequence of crosses made between different botanical types in the course of breeding new improved groundnut varieties, several intermediate types having the specific traits of more than one botanical type are now under cultivation across the world. Hybridization between two botanical types can break linkages between the traits, which have otherwise co-segregated for over centuries to form distinct botanical types.

5.2 Breeding Methodologies

5.2.1 Mode of Reproduction and Artificial Hybridization

Groundnut is a self-pollinated crop, but natural hybridization can occur where bee activity is high (Nigam et al. 1983). Flowering begins 17–35 days after seedling emergence depending on the cultivar and environmental conditions. In var.

hypogaea and var. *fastigiata*, the inflorescence is simple and that of var. vulgaris is compound. Flowers are born in the axils of the leaves and never at the same node as vegetative branch. One or more flowers may be present at a node. The flower consists of five petals, ten monadelphous stamens, two of which are not fully developed while the other eight are fertile, and a pistil. Among the eight fertile anthers, four are globose and the other four are oblong type. The pistil consists of ovary, style and stigma. The ovary contains a single sessile carpel and one to six ovules. The style is glabrous throughout its length and covered with bristles near the club-shaped stigma. The stigma becomes receptive to pollen about 24-h before anthesis and remains so for about 12 h after anthesis, and the dehiscence of anthers takes place 2–3 h prior to opening of the flower in the morning. Fertilization occurs about 6 h after pollination. In a week after fertilization, the peg or gynophore carrying the ovary and fertilized ovule grows and enters the soil where the pods develop. The tip orients itself horizontally away from tap root (diageotropic).

Emasculation of groundnut can be accomplished on warm bright days between afternoon and evening. A well-developed bud is selected for emasculation, and all the other buds at the node are removed mechanically. The selected bud is carefully opened and the anthers are fully removed. A small coloured thread is tied on the node of the emasculated flower for identification at the time of pollination next morning. A healthy flower from the male parent (pollen source) is plucked and the pollen is gently squeezed on to the stigma of the emasculated flower or, alternately, the pollen is squeezed on to forceps, and then transferred to the stigma of emasculated flower. The maximum physiological development of pollen is in the early hours of the day. It was observed that pollen remained viable up to 8 days when stored in a sealed desiccator with calcium chloride in a refrigerator at 6°C. Hybridizing groundnuts in the greenhouse may result in over 70 % success, higher than that obtained in field. The procedure for hybridization in groundnut has been described in detail by Nigam et al. (1983).

5.2.2 *Breeding Methodologies*

Several reviews describing breeding methodologies in groundnut have been published (Wynne and Gregory 1981; Isleib et al. 1994; Knauft and Wynne 1995) and a large number of cultivars following these methodologies have been released across the world that not only have high yield potential but possess resistance/tolerance to biotic and abiotic stresses and have improved quality traits (for oil and food uses). ICRISAT has contributed to the release of 138 groundnut cultivars between 1986 and 2010 through its partners in National Agricultural Research System (NARS) in 36 countries of Asia and Africa. The breeding methods used for self-pollinated crops are applied in groundnut breeding. They include mass selection, pedigree, bulk, single seed descent and back-cross methods.

5.2.2.1 Introduction and Mass Selection

Like in any other crop, at initial stages of a breeding program, introductions played an important role in groundnut also as they are either directly used as cultivars or Mass-selection is practiced in introduced genotypes to develop a new cultivar. JL 24, a popular short-duration groundnut cultivar in India, is a classic example for selection made in the material, EC 94943, introduced from Taiwan. The selection, made at the Oilseeds Research Station, Jalgaon, Maharashtra, was released as JL 24 (Phule Pragati) in 1979 for cultivation in India (Patil et al. 1980). Subsequently, it was introduced into Africa and was released in several countries there.

5.2.2.2 Hybridization and Handling of Segregating Populations

To combine traits from different parents in improved groundnut cultivars, the parents, selected for desirable traits are hybridized followed by selections for desirable trait combinations in segregating populations. Single crosses, three-way crosses, and double crosses are also used to derive segregating populations. Multiple crossing systems, such as the convergent cross, to create adequate genotypic variability before selection (Wynne and Gregory 1981) were also used. In groundnut, pedigree and bulk-pedigree methods of breeding are most frequently used to handle segregating populations derived from hybridization. Confirming the hybridity of F_1 plants based on the morphological characteristics and pod and kernel features is important in groundnut and it is done by growing male and female parents along with F_1's. Since seed multiplication rate is low in groundnut (1:10), it is advisable to make large number of pollinations to get sufficient number of hybrid seeds to generate large enough F_2 population. In pedigree method individual plants are selected in F_2 population and F_3 progeny rows are grown in the next season. Selection of single plants is continued in F_3 and F_4 progeny rows. More than one individual plant is selected and bulked from best progenies in F_5 generation and repeated in F_6 generation. The F_7 generation is advanced to either preliminary yield trials or seed increase, if sufficient seeds are not available for including in trials. Bulk-pedigree method, aimed at improving traits with low heritability, is a modified method of bulk method in which individual plants of F_2 are harvested in bulk up to F_4 generation and then single plant selections are made and subsequent generations are handled as in pedigree method (Wynne and Gregory 1981).

Another modified bulk method of selection is using single-seed descent method. The main advantage of this procedure is that the characters with low heritability can be improved as the genetic variance for these traits is maintained. Single seed decent method is becoming popular as this has the advantage to save space and resources (Isleib et al. 1994). In single-seed decent method, one or two seed from each plant of F_2 and F_3 are advanced and in F_4 generation single plant selection is done and raised as individual plant progenies in F_5 generation. The handling of material from here is similar to that of pedigree method. In the recent times, with the advent of molecular markers linked to the traits of interest and QTL identification and

mapping, backcrossing is used frequently in breeding programs. In fact, Marker Assisted Backcrossing (MABC) is the most frequently used method of breeding in groundnut to transfer a desired allele or QTL into the target genotype (recurrent parent) (Chu et al. 2011).

5.2.2.3 Sources of Variability

The cultivated accessions of *Arachis* in gene banks across the world and the advanced breeding lines available with the breeder are often used as parents in breeding programs and hence serve as important sources of variability. Induced mutants and interspecific derivatives are other important sources of variability. The gene banks are also the repositories of wild *Arachis* species and interspecific derivatives. The gene bank at ICRISAT, India has the largest collection of groundnut genetic resources that include 14,310 accessions of *Arachis hypogaea* and 413 accessions of wild *Arachis* species (Upadhyaya et al. 2001). The other large collections are available at United States Department of Agriculture (Holbrook 2001), Texas A&M, North Carolina State University, National Center of Genetic Resources (CENARGEN) in Brazil (Holbrook and Stalker 2002), National Bureau of Plant Genetic Resources (NBPGR) in India, and Chinese Academy of Agricultural Sciences (Boshou and Holbrook 2007).

5.2.2.4 Population Improvement

Population improvement procedures are not commonly used in groundnut as it is a highly self-pollinated crop with cleistogamous flowers. Nevertheless, diallel selective matings and modified recurrent selection schemes were applied to a limited extent in groundnut improvement (Wynne 1976) that led to development of some higher yielding groundnut cultivars with a broad genetic base (Monteverde-Penso et al. 1987). The difficulties involved in making large number of pollinations have limited the use of recurrent selection schemes although its potential was identified to improve several traits in groundnut.

5.2.2.5 Mutation Breeding

The above described methods of breeding enable reshuffling of the existing variability and fixing the desirable combinations, while through induced mutations new variability is created. Mutation breeding has also been extensively used in groundnut breeding. Mutation breeding is often used to improve a superior breeding line or a popular cultivar for a single-specific trait such as, bold kernel size, disease resistance etc. Both physical and chemical mutagens have been used in groundnut to induce mutations. Under its joint FAO-IAEA program, about 72 groundnut varieties were developed through mutation breeding and released for cultivation

worldwide over the last five decades (http://mvgs.iaea.org). By 1996 in China, 14.7 % of new groundnut varieties were bred from induced mutants and they accounted for 19.5 % of the cumulative cultivated area in China (Qui et al. 1998). In India, the Nuclear Agriculture and Biotechnology Division of the Bhabha Atomic Research Centre, Mumbai has developed cultivars using either mutation breeding or a combination of mutation and hybridization (Kale et al. 1999, 2000).

5.2.2.6 Wide Hybridization

Unlike cultivated groundnut, wild *Arachis* species are reported to possess high levels of resistance to rust, leaf spots, nematodes, peanut bud necrosis virus (PBNV), tobacco streak virus (TSV), tomato spotted wilt virus (TSWV), groundnut rosette virus (GRV), groundnut rosette assistor virus (GRAV), leaf miner, *Spodoptera*, jassids, thrips, aphids and abiotic stresses (Dwivedi et al. 2003; Rao et al. 2003; Nautiyal et al. 2008; Kalyani et al. 2007; Dwivedi et al. 2008). Wide hybridization has been used to expand the available variation using wild species and several interspecific derivatives have been developed for use as donors of desirable traits or released as cultivars. Synthetic amphidipoids of *Arachis* sp. can be useful to develop wild introgression lines in cultivated background (Fonceka et al. 2009) and can facilitate better utilization of wild species in breeding programs as use of synthetic amphidiploid circumvents the crossing barrier between wild and cultivated species of *Arachis* (Fonceka et al. 2009; Mallikarjuna et al. 2010). Synthetic amphidiploids have been bred at ICRISAT using different combinations of wild *Arachis* including the progenitors of cultivated groundnut, *A. duranensis* and *A. ipanensis* (Mallikarjuna et al. 2010).

5.2.3 Marker Technologies and Genetic Transformation

Although low genetic diversity in cultivated groundnut gene pool was a serious bottleneck until recently in developing the genetic maps based on mapping populations of cultivated groundnut lines, availability of large number of simples sequence repeat (SSR) markers facilitated the development of the first SSR-based genetic map based on recombinant inbred line (RIL) population derived from TAG 24×ICGV 86031 (Varshney et al. 2009a). With an objective of estimating the marker order for a maximum number of marker loci based on a single map, a composite map comprising of 175 marker loci has been developed by Hong et al. (2010). Although several genetic maps have become available for cultivated groundnut, single nucleotide polymorphic (SNP) markers have not yet been integrated into these maps. Linked markers for nematode resistance (Burrow et al. 1996; Garcia et al. 1996), aphid vector of groundnut rosette disease (Herselman et al. 2004), yield and yield parameters (Selvaraj et al. 2009), drought tolerance related traits (Varshney et al. 2009b; Ravi et al. 2011), resistance to foliar disease (Khedikar et al. 2010) and

nutritional quality traits (Sarvamangala et al. 2011) were identified. Following marker assisted breeding, "Tifguard High O/L" cultivar was developed through three rounds of accelerated backcrossing to pyramid nematode resistance and the trait for high oleic:linoleic acid (high O:L) ratio in seeds (Chu et al. 2011). At ICRISAT, marker assisted backcrossing is underway for transfer of QTLs conferring resistance to foliar fungal diseases. Pandey et al. (2012) reviewed the development of genomic resources such as development of molecular markers, genetic and physical maps, generation of expressed sequenced tags (ESTs), development of mutant resources, and functional genomics platforms that facilitate the identification of QTLs and discovery of genes associated with tolerance/resistance to abiotic and biotic stresses and agronomic traits.

Reports of the transformation and development of groundnut transgenics using a variety of genes such as the bar gene for herbicide tolerance (Brar et al. 1994), cry1A (Singsit et al. 1997), a chimeric Cry1X gene (Entoori et al. 2008), a gene encoding the nucleocapsid protein of the tomato spotted wilt virus (Yang et al. 1998), a gene that confers resistance to Indian peanut clump virus, gus (Venkatachalam et al. 2000; Rohini and Rao 2000), chitinase (Rohini and Rao 2001), DREB1A for drought tolerance (Bhatnagar Mathur et al. 2007), non-heme chloro-peroxidase gene (cpo-p) from *Pseudomonas pyrrocinia* having antifungal activity (Niu et al. 2009), and AtNHX1, a gene driven by 35S promoter for salt and drought tolerance (Asif et al. 2011) have been reported. Recent developments in the area of transgenic research through modification of aflatoxin biosynthesis pathway or use of genes with antifungal and anti-aflatoxin properties also appear to be encouraging. A post-transcriptional gene silencing (PTGS) model to knock out the production of allergenic protein, Ara h 2 in groundnut by specific degradation of the endogenous target messenger RNA (mRNA) was demonstrated (Dodo et al. 2005). Groundnut allergy is an IgE-mediated hypersensitivity reaction. ICRISAT has developed a pipeline of genetically engineered groundnuts for several traits that are in different stages of product development including pathogen-derived resistance to viruses, anti-fungal genes for resistance to fungal pathogens and aflatoxin contamination, nutritional enhancement by the over-production of β-carotenes and for tolerance to drought stresses.

5.3 Breeding Objectives and Phenotyping

Developing new varieties with increased yield potential and resistance to biotic and biotic stresses that meet the needs of the growers, processors and consumers is the primary objective of groundnut breeding. With the constraints that limit the yield potential and emerging market and consumer preferences, groundnut breeders always have a challenging task to breed new genotypes to meet these requirements. Once the objectives are defined, the traits that meet the objectives will be identified. Phenotyping the target trait is an important aspect in any breeding program. Appropriate trait phenotyping enables the breeder to make desirable selections in

segregating generations and advance the breeding lines in yield trials. In this section, we discuss various phenotyping tools to qualify/quantify traits that meet various objectives of breeding groundnut. Phenotyping may not be possible for all the traits that are targeted in a breeding program due to their complex nature, in such cases, simple surrogates, if known, could be used.

5.3.1 Maturity Duration

Regions of groundnut cultivation have varying lengths of growing period (LGP) (90 to over 150 days); based on the LGP, three maturity groups are identified, early or short (90–110 days), medium (110–130 days) and late (over 130 days). Temperature plays a critical role in determining duration of maturity; higher temperature reduces the crop duration, while reverse is true at lower temperatures. Early maturing varieties are important in various agroecological regions like (1) rainfed semi-tropics where growing season is short (100–110 days), and/or end of season drought is frequent, a typical scenario in South India, (2) irrigated with short cropping window in multiple cropping systems, a typical situation in South East Asia (<100 days), and (3) rice fallows, where crop is grown on residual moisture (<100 days). Further, breeding for short-duration in groundnut has become more relevant with the predicted decline of LGP by 5 % or more across the tropics by the climate change, agriculture and food security (CCAFS) research.

As the "calendar days" concept of determining maturity duration is location and season specific, it cannot be applied in breeding for short-duration varieties for other locations or for the same location over years. As growth and development in groundnut is largely driven by temperature (Ong 1986), the concept of cumulative thermal time or degree days (CTT or °Cd), which is both, location and season neutral, was developed at ICRISAT to breed short-duration varieties with stable maturity duration across locations (Vasudeva Rao et al. 1992). Taking 10 °C as base temperature, CTT or °Cd (cumulative thermal time or cumulative degree days) for each day is calculated from maximum and minimum temperatures. The daily thermal time is accumulated each day from planting to harvest to arrive at the CTT or °Cd. At ICRISAT location, the standard CTTs for the 75- and 90-day crop growing periods is 1,240 and 1,470 °Cd, respectively. These standard CTTs are used to predict the harvest dates for early-maturing groundnut variety trials and breeding materials in each season. The CTT or °C d for other locations can also be worked out using the formula: ${}_{H}\sum^{P}\{[({}^{T}\text{max} * {}^{T}\text{min})/2]-{}^{T}\text{base}\}$, where ^{T}max is daily maximum temperature, ^{T}min is daily minimum temperature, ^{T}base is mean base temperature for groundnut, P is planting date and H is harvest date. In breeding for short-duration, both, segregating progenies and preliminary and advanced yield trails are harvested at 1,470 °Cd and selection is practiced based on number of mature pods per plant and yield. The selection is more intense in elite trial that is staggered harvested twice, first when the crop is exposed to 1,240 °Cd and next at 1,470 °Cd. The selection for early maturity is based on the increase in pod yield, shelling outturn

and 100-seed weight from 1,240 and 1,470 °Cd, and the genotypes recording the least increase are selected. For breeding medium and late duration varieties, both, the segregating generations and the entries in yield trials are harvested when the plants show the signs of physiological maturity and selections are carried out based on yield and yield attributes including percentage of sound mature kernels. As the breeding for multiple traits is becoming common practice, resistance to biotic and abiotic stress, quality and other consumer and market preferred traits are targeted along with the duration.

5.3.2 Yield and Yield Attributes

Selection for yield has been the basis for improving groundnut productivity in the world (Nigam et al. 1991), but gains from such selection are slow due to large environmental effects. Yield and yield attributes are quantified for appropriate selection and advancement of both, segregating progenies and test entries (advanced breeding lines). In segregating generations, selection and advancement for yield and yield attributes is, in general, qualitative, but in yield trials, conducted to test the performance of advanced breeding lines, yield attributes are measured (quantified) in replicated trials following appropriate field designs and statistical procedures. In segregating generations, for both individual plant or bulk selections, visual observations on growth habit, branching pattern, yield and yield attributes, pod and seed characteristics, proportion of pod yield to the total biomass, in-situ germination (due to lack of dormancy), peg strength etc. are taken into account for generation advance. But in yield evaluation trials, the specific observations are recorded on: days to 50 or 75 % emergence in a plot, initial and final plant population in a plot, days to flowering (initiation, 50 or 75 % plants in a plot flowering), growth habit, branching habit, reaction to diseases and insect pests, individual plant observations (number of primary and secondary branches, height of main axis and length of primary branches, number of pods, pod yield and seed yield per plant), days to maturity, plot pod and haulm yields, shelling outturn, 100-seed weight, pod characteristics (size, number of seeds per pod, reticulation, beak and constriction), seed size, shape and color and fresh seed dormancy etc. Depending upon the objectives of the trial, appropriate observations on yield/yield attributes are selected for recording data.

Replicated yield trials are conducted to determine the performance of advanced breeding lines. Each breeding program may have its own protocol of evaluation of advanced breeding lines for yield and other agronomic traits. At ICRISAT, the performance of advanced breeding lines is evaluated following a three-tier system of evaluation that includes trait-specific preliminary, advanced and elite trials organized based on growth habit. Test entries (advanced breeding lines) are first compared with appropriate controls in smaller plots in preliminary trials organized based on growth habit (Spanish or Virginia bunch) and conducted in both rainy and postrainy season. Based on performance in two seasons, the promising entries are promoted to the next level of trials—advanced trials. The entries at this

level are evaluated in a larger plot size in both rainy and postrainy season. The best performing entries from advanced trials are promoted the elite trials which are again evaluated for two seasons in bigger plots. Based on the performance over six seasons (3 years), the selected entries are identified for inclusion in international trials which are made available to National Agricultural Research System (NARS) partners on request. The promising entries from international trials and from their own breeding programs are evaluated in multilocation trials at the state or national level. Multilocation yield trials allow breeders to identify location specific and/or widely adapted stable varieties for release, assess the stability of resistance/tolerance to biotic and abiotic stresses and estimate G×E for different traits of economic importance. To avoid confounding effects of the phenology of the crop, the comparison of test entries with controls are done within a taxonomic group. Maintaining optimum plant population in all the test entries and controls is an important aspect in the conduct of yield trials, as plant population is directly correlated with pod yield and there is little compensation mechanism for the low plant stand/missing plants in groundnut.

5.3.3 Resistance Breeding

The potential and realized yields are represented in Fig. 5.2 (adapted from Johansen and Nageswara Rao 1996), where yield potential is defined as the maximum yield obtainable by the best genotypes in a specified agroclimatic environment when the known biotic and abiotic constraints are overcome. From Fig. 5.2, it is apparent that the yield gap, the difference between yields realized by farmers and potential yield, is large for major producing countries or regions. It implies that there is considerable scope for increasing yield by identifying genotypes resistant to biotic and abiotic stresses and/or by addressing these constraints through management options. The phenotyping tools useful in breeding varieties for tolerance/resistance to abiotic and biotic factors are discussed in the following sections.

5.3.3.1 Abiotic Stress

Drought and high temperature are the most important abiotic stresses that are widespread in groundnut growing areas. The others include salinity and acid soils. Drought is a major abiotic constraint in the semi-arid tropics affecting yield and quality in groundnut. Yield losses due to drought depend on its timing, intensity and duration. Depending on the time of occurrence, drought can be characterized as early season, mid-season and end-of-season drought. An annual estimated loss in groundnut production equivalent to US$520 million (at the market prices of 1994) is caused by drought. Almost half of it (US$208 million) can be recovered through genetic enhancement for drought tolerance with a benefit: cost ratio of 5.2 (Johansen and Nigam 1994). Further, drought predisposes pre-harvest *Aspergillus* infection in

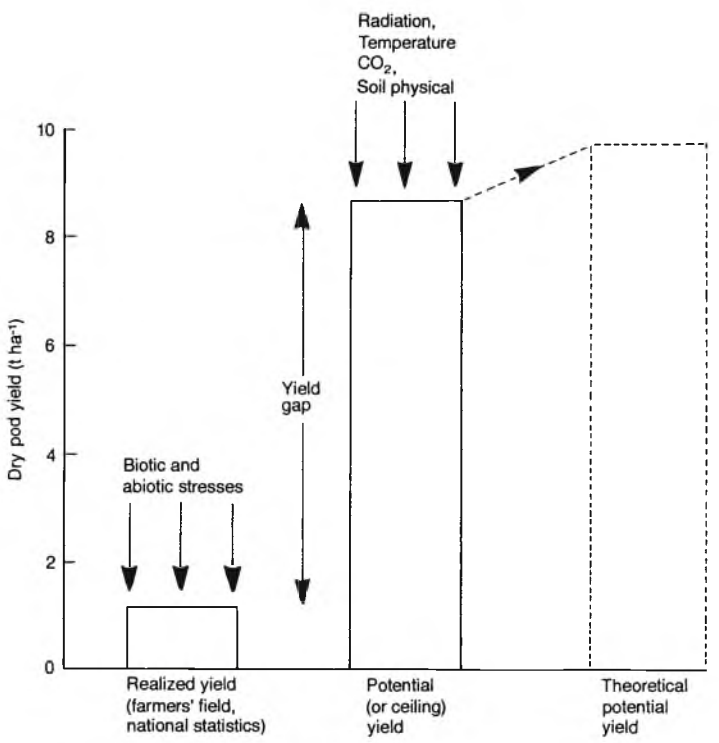

Fig. 5.2 Representation of realized and potential yields and their relationships (*source*: Johansen and Nageswara Rao 1996)

the field that affects quality of produce. Linked closely with drought is high temperature stress. The CGIAR's climate change for agriculture and food security (CCAFS) research has shown that high temperature stress (above 30 °C) will be widespread in East and Southern Africa, India, South East Asia and Northern Latin America, which are important groundnut growing areas.

5.3.3.2 Phenotyping for Drought Tolerance

The breeding approaches include developing short-duration varieties to escape end-of-season drought and drought tolerant varieties that can withstand moisture stress through various mechanisms. Both, empirical (also called conventional) that involve selection for superior yield performance under drought conditions and trait-based approaches are used in breeding. Studies have shown that both these approaches led to same gains in breeding for drought tolerance (Rao and Nigam 2003). In empirical approach, genotypes tolerant/resistant to drought are identified by assessing their total and pod dry matter productivity under drought stress (Rao and Nigam 2003). The evaluations are done in both well watered and imposed water stress conditions

and genotype with superior pod and biomass yield in well watered conditions along with least reduction in pod and biomass yield under water stress are selected. For such evaluations in rainy season, supplemental irrigation is provided during dry spell for well watered treatment, while for stress treatment no irrigation is provided. However, the differences get nullified when the rainfall in the rainy season is well distributed; hence postrainy (or dry) season results are more reliable where stress is imposed by withholding of scheduled alternate irrigations from 60 DAS up to harvest and there is less interference of rainfall. The empirical approach is both labour and resource intensive; nevertheless it is most extensively used. At ICRISAT and elsewhere in national programs of Asia, Africa and the USA (Branch and Kvien 1992), breeders have been successful in developing drought tolerant groundnut genotypes using empirical approach. ICGV 91114, a drought tolerant groundnut variety developed at ICRISAT and released in India in 2006, is slowly replacing old and less productive varieties in highly drought prone district of Anantapur (Birthal et al. 2011) in Andhra Pradesh state. When a larger number of genotypes have to be screened, line source sprinkler technique can be used that evaluated genotypes under varying intensities of drought and empirical approach is used for evaluating the performance of the genotypes. However, strong winds and rains influence this technique and it requires complex statistical analysis (Singh et al. 1991).

Trait-based approach involves phenotyping for the traits like transpiration, transpiration efficiency (TE), water use efficiency (WUE) and harvest index and it is expected that genotypes selected for these traits will have stable yields across erratic rainfall. Measurement of WUE and TE that requires special growing facilities such as rainout shelter and lysimeters to grow plants under controlled water regimes are not often used in breeding programs. WUE is determined by gravimetric approach that involves determination of total water transcribed by a plant over a specific period of crop growth and the total biomass the plant accumulated over the same period. For this the plant are grown in suitable containers that are weighed once or twice daily and the difference on subsequent days is corrected by adding an extra amount of water. As a whole plant, WUE can be determined during the period between 25 and 65 days after sowing. TE is also assessed gravimetrically by growing the genotypes in lysimeters under well-watered or drought conditions. Transpiration is measured by regularly weighing the lysimeters, in which the soil surface is mulched with a 2-cm layer of polythene beads to avoid water evaporation from the soil (Ratnakumar et al. 2009).

As it is cumbersome to measure WUE and TE in an applied breeding program, instead its surrogates such as specific leaf area (SLA) and SPAD chlorophyll meter readings (SCMR) are used. "Carbon isotope discrimination" is an important surrogate to WUE/TE but determining is cumbersome, expensive and sometimes not correlated to increased yield and hence not often used in breeding programs (Sheshashayee et al. 2003). SLA is obtained by dividing the area of a fresh leaf by its oven-dry mass, expressed in $m^2\ kg^{-1}$. Leaf area meter is used to determine the leaf area and the samples are dried in an oven for 2 days at 70 °C to obtain the oven-dry mass. A direct close relationship of TE with SCMRs was reported in groundnut (Rao et al. 2001) and SCMR also has a direct linear relationship with extracted leaf

chlorophyll and leaf nitrogen concentration. The advantages such as easy and rapid measurement, nondestructive method and light weight made SPAD chlorophyll meters the best choice for use in the trait-based groundnut breeding programs (Serraj et al. 2004). The SCMRs are recorded on the second or third leaf from the top that is completely expanded and two readings covering on either side of midrib are taken on each leaflet and the average SCMR is computed. Care is taken to cover the SPAD meter sensor with leaf lamina and interface with midrib and veins are avoided to improve the accuracy of the readings. At ICRISAT both empirical and trait based approaches are used; while selection in segregating generations is based on total dry matter and pod yield under stress, in yield trials, in addition to these, SCMR and SLA are also used as selection criteria.

5.3.3.3 Screening for High Temperature Tolerance

A common method of selecting plants for heat-stress tolerance is to grow breeding lines in a hot target production environment and identify individuals that do not compromise on pod yield and quality at elevated temperatures (Ehlers and Hall 1998). Heat tolerance screening in glasshouses may be a more effective method as screening can be carried out throughout the plant life cycle, from seedling to reproductive stages. Better control of temperatures and other experimental parameters in glasshouse are additional advantages. Breeding for heat tolerance in groundnut is at infancy and the best screening method and selection criteria are yet to be identified. Two key stages, flowering including microsporogenesis (3–6 days before flowering), and fruit-set were measured to assess tolerance to high air temperature in groundnut (Craufurd et al. 2002, 2003). Vara Prasad et al. (1999) showed that 34 °C is threshold temperature for pollen production in groundnut. Membrane thermostability is also used to evaluate the genetic variability for heat stress in groundnuts by using the heat killing temperature and heat killing time as the selection index (Talwar et al. 2002).

5.3.3.4 Screening for Tolerance to Salinity

Screening for salinity tolerance can either be done under controlled conditions (pot culture method) or in the field. However, each of the two methods has inherent limitations; while screening under controlled conditions raises the question of its applicability in the field, the experimental error is huge under field conditions. Since a combination of both the methods was suggested for efficient screening, a screening protocol was standardized using 100–125 mM of NaCl water under a facility that allows both a rigorous control on salt treatment and yield evaluation (Vadez et al. 2005). Such a facility, located outdoors, is available at ICRISAT and is equipped with moveable rainout shelters and uses large pots filled with natural soil. Salt application is made on a per unit soil basis dissolved in irrigation water to ensure uniform distribution. In addition to measuring reduction in yield under salt stress, the other

key indicators under salinity stress are: large stem proportion that may serve as sodium sink to confer tolerance, maintaining the leaf size and relatively less reduction in nodulation. Singh et al. (2008) screened groundnut materials in two consecutive seasons, first by imposing the salinity treatment as irrigation with saline water (6–7 dS m^{-1}) during summer season and then on residual salinity in next season. The salinity was build up to a range of 4.0–8.0 EC measured regularly during cropping season.

5.3.3.5 Screening for Tolerance to Aluminum Toxicity

At high soil acidity, it is not usually the hydrogen ion activity that limits plant growth but rather the toxicity and deficiencies of elements. Aluminum toxicity is the single most important factor that effects plant growth and yield under acid soils. Pot experiments were conducted by adding $AlCl_3$ (40 mg Al l^{-1}, pH 4) to the soil in a pot to create Al toxic conditions before sowing (Boshou et al. 2000) and reduction in yield under Al toxicity is measured to determine tolerance to acid soils. The primary response to aluminum stress occurs in the roots and it was shown that root dry weight per plant, root volume per plant and shoot dry weight per plant were the key indicators for evaluating Al tolerance. The concept of "average Al tolerance coefficient" for the evaluation of Al tolerance in groundnut genotypes was also put forth (Boshou et al. 2000). Solution culture using $AlCl3.6H_2O$ @ 5–15 mg/l (Yang and Jing 2000) or 40 ppm aluminum solution prepared using $Al_2(SO_4)_3{\cdot}6H_2O$ (Pratap et al. 2002) and soil block culture and field experiments (Yang et al. 1998) were also used to study varietal responses to Al toxicity. Field evaluations following duplicate tests, one on natural un-reclaimed acid soil and other lime-amended plot is desirable.

5.3.3.6 Fungal Diseases

Groundnut is attacked by several diseases caused by fungi of which foliar fungal diseases, *Aspergillus* infection and pod and stem rot are widespread and important. Late leaf spot (LLS) caused by *Phaeoisariopsis personata* (Berk. & Curt.) Van Arx, early leaf spot (ELS) caused by *Cercospora arachidicola* Hori and rust caused by *Puccinia arachidis* Spegazzini are among the major foliar fungal diseases. Both leaf spots are commonly present in all groundnut growing areas but the severity of each disease varies between locations and seasons. An estimated global yield loss of US$600 million due to LLS was reported (Dwivedi et al. 2003). At ICRISAT, breeding for foliar fungal disease has resulted in development of several genotypes with high level resistance to rust and moderate resistance to LLS (Singh et al. 2003). There is scope to further enhance the level of resistance to these diseases. Aflatoxins are potent carcinogen produced by *Aspergillus* infection forcing several countries to have strict regimes in place on permissible levels of aflatoxins in their imports of groundnuts. *Aspergillus flavus* is predominant in Asia and Africa, while *Aspergillus parasiticus* is the predominant species in America. Lamb and Sternitzke (2001)

estimated that aflatoxin contamination costs over $20 million in losses to the southeast U.S. groundnut industry. Stem and pod rot, caused by *Sclerotium rolfsii* is a potential threat to groundnut production in many warm, humid areas, especially where irrigated groundnut cultivation is expanding.

5.3.3.7 Screening for Foliar Fungal Diseases

High yield potential and high degree of resistance do not generally go together (Nigam et al. 1991). In most breeding programs a balance is struck between these two traits—combining high yield potential with moderate levels of resistance to avoid penalty in yield potential. Advanced breeding lines and segregating generations are screened in a disease screening nursery under infector row system during rainy season and selections are done for both, resistance to disease and superior yield under disease pressure (Tallury et al. 2009). Both, field and controlled conditions screening can be used, although field screening is widely used in breeding programs.

Field Screening for Leaf Spots and Rust. Different infector row arrangements are practiced for advanced breeding lines and segregating populations. Segregating population are generally grown in ridge and furrow system. After every 5–10 rows of segregating populations, an infector row (a mixture of short- and medium-duration, highly susceptible to foliar diseases varieties) is planted. The frequency of occurrence of infector rows depends on the location and season of foliar diseases screening. The screening block is surrounded by rows/plots of infector rows on all sides. To verify uniform disease spread, plots of a foliar diseases susceptible variety are also interspersed along with segregating populations. Screening of advanced breeding lines is done in replicated plots along with susceptible controls in broad bed and furrow system. After every fourth bed of test material, a bed of mixture of susceptible varieties forming infector row is also sown. Infector rows/beds are inoculated with a conidial (for leaf spot)/urediniospore (for rust) suspension at flowering stage. If needed, the inoculation can be further repeated. In addition, the artificially inoculated potted "spreader" plants are also placed throughout the field to serve as an additional source of inoculum. After inoculation, perfo-irrigation is provided daily for 15 min in the evening hours for 30 days to favour building up of humidity required for disease development. A 9-point scale, as given by Subrahmanyam et al. (1995), is followed for scoring for leaf spots and rust reaction in the field (Table 5.1). The genotypes recording a score of 1–4 are considered to be resistant. Disease scoring is done two to three times at intervals depending up on the requirement. Yield and yield contributing traits are also recorded in yield trials for making selections based on both disease reaction and yield under disease pressure.

Detached Leaf Technique for Leaf Spots and Rust. Detached leaf method is a rapid technique for screening resistance to leaf spots (Foster et al. 1980) and rust (Mayee and Munde 1979) in groundnut. Detached, healthy groundnut leaves rooted in sterile sand in trays are inoculated with a concentration of 30,000 spores ml^{-1} for LLS and 10^5 urediniospores ml^{-1} for rust, followed by incubation in the growth chamber

Table 5.1 A 1–9 scale for recording reaction of foliar diseases in groundnut in the field (Subrahmanyam et al. 1995)

Disease score	Description	Disease severity (%)
(A) Late and early leaf spot diseases		
1	No disease	0
2	Lesions present largely on lower leaves; no defoliation	1–5
3	Lesions present largely on lower leaves, very few on middle leaves; defoliation of some leaflets, evident on lower leaves	6–10
4	Lesions on lower and middle leaves, but severe on lower leaves; defoliation of some leaflets, evident on lower leaves	11–20
5	Lesions present on all lower and middle leaves; over 50 % defoliation of lower leaves	21–30
6	Severe lesions on lower and middle leaves; lesions present but less severe on top leaves; extensive defoliation of lower leaves; defoliation of some leaflets, evident on middle leaves	31–40
7	Lesions on all leaves but less severe on top leaves; defoliation of all lower and some middle leaves	41–60
8	Defoliation of all lower and middle leaves; severe lesions on top leaves; some defoliation of top leaves evident	61–80
9	Almost all leaves defoliate, leaving bare stems; some leaflets may remain, but show severe leaf spots	81–100
(B) Rust disease		
1	No disease	0
2	Pustules sparsely distributed, largely on lower leaves	1–5
3	Many pustules on lower leaves, necrosis evident; very few pustules on middle leaves	6–10
4	Numerous pustules on lower and middle leaves; severe necrosis on lower leaves	11–20
5	Severe necrosis of lower and middle leaves; pustules may be present on top leaves, but less severe	21–30
6	Extensive damage to lower leaves; middle leaves necrotic, with dense distribution of pustules; pustules on top leaves	31–40
7	Severe damage to lower and middle leaves; pustules densely distributed on top leaves	41–60
8	100 % damage to lower and middle leaves; pustules densely distributed on top leaves	61–80
9	Almost all leaves withered; bare stems seen	81–100

at 24 °C temperature and 85 % relative humidity and a 12 h light/12 h dark regime. LLS disease development is determined every 2 days from 5 to 37 days after inoculation and observations are recorded on incubation period (days), latent period (days), lesion number, lesion diameter (mm) and leaf area damage (%) (Janila et al. 2013). Rust pustules appear some 10 days after inoculation on susceptible genotypes. Disease severity is scored on a 1–9 scale, where 1 = no disease, and 9 = 81–100 % foliage destroyed (Subrahmanyam et al. (1983). The other components that the recorded include incubation period (days), infection frequency (lesions per cm^2), pustule diameter (mm), pustule ruptured (%), spores per mm^2 of

pustule area and Urediniospore germination (%). Incubation period is days from inoculation to appearance of first lesion/pustule, and latent period is days from inoculation to the appearance of first sporulating lesion/ruptured pustule. Lesion/pustule diameter is measured using vernier caliper under a magnifying glass. Leaf area damage as percent is assessed by comparing the leaves with diagrams depicting leaves with known percentage of their areas affected (Hassan and Beute 1977). Urediniospores per unit area and germination are measured under a microscope.

Screening for Aspergillus Infection and Aflatoxin Contamination. The infection of *Aspergillus* can occur before harvest in the field, during post-harvest drying and curing and in storage. Infection can result in aflatoxin contamination in groundnut kernels. Resistance to *Aspergillus* in groundnut operates at three independent sites, pods, seed coat and cotyledons (Utomo et al. 1990). A three-step evaluation is adopted at ICRISAT for screening: pre-harvest infection (Mehan 1989), in vitro seed colonization and aflatoxin production (Mehan and McDonald 1980). Holbrook et al. (1994) developed a large scale field system for screening groundnut germplasm for resistance to aflatoxin contamination at Yuma, Arizona as a screening site because it consistently has hot and dry conditions.

Field screening for pre-harvest infection involves growing the genotypes in replicated trials in an *Aspergillus flavus* sick plot and imposing drought late in the season to promote the infection (Mehan 1989) as drought stress predisposes *Aspergillus* infection. Postrainy/dry season allows creation of moisture stress conditions in the field without interference from rains by withholding irrigations from 60 to 70 days after sowing up to harvest. To ensure sufficient inoculum load at the pod zone, soil inoculation is repeated three to four times from 25 days after flowering. At ICRISAT, a highly toxigenic *A. flavus* isolate AF 11-4 is multiplied on autoclaved sorghum seed in the conical flasks. After 5 days, the inoculum is removed from the flasks and mixed with autoclaved sorghum seed (1:5 ratio) before the field application. For inoculum application, the soil near the groundnut plants is opened about 3–5 cm deep on either side of the plants in a row. The mean soil temperature of 29–31 °C in the podding zone is preferred. The pods are harvested at maturity and carefully shelled. The shelled kernels are tested in laboratory for pre-harvest seed infection by incubating them in petri plates at 99 % relative humidity.

Screening of groundnut genotypes can also be done by investigating in-vitro seed invasion and colonization and aflatoxin contamination (IVSCAF), an indicative of resistance at the sites of seed coat and cotyledon, respectively. To study IVSCAF, the seeds are inoculated with *A. flavus* and incubated for seed invasion and colonization is recorded as incidence percentage. The seed sample is prepared either by scarification or removal of testa. Then they are placed on the surface sterile filter paper moistened with 5 ml of sterile water in a 10-cm plastic petri dish and inoculated with 25 μl of a suspension containing approximately 1×10^6 conidia per ml of *A. flavus* and incubated at 28 °C. After 8 days, samples were removed from the incubator and rated separately for mycelial growth, green color, and development of "fluffy" colonies on a proportional scale of 0 (no growth, green color, or fluffy colonies) to 10 (dense mycelium on all quarters, dark green color, or all fluffy colonies) in one-point increments (Xue et al. 2003). Since it is the aflatoxin contamination and not the

Aspergillus infection itself which is important, estimation of aflatoxin concentration seems to be the best technique to screen genotypes for tolerance. Hence aflatoxin contamination of genotypes is estimated for both, pre-harvest and in vitro infected seed samples. The cotyledons are tested for aflatoxin contamination using enzyme-linked immunosorbent assay (ELISA). Commercial kits are also available in the market such as, Aflatest, Agriscreen, Aflacup 10, Aflacup 20 and EZ-Screen with 1–20 ppb detection power (www.oxnet.ksu.edu/grsiest). For selection of genotypes, it is plot-wise data and not mean over the replications that is taken into consideration. For instance, the genotypes with low infection/contamination in all the replication are selected, while those with low value in one replication and high in other are rejected. In China, several new groundnut cultivars with improved productivity and resistance to aflatoxin contamination are extensively used in production. In addition, integrated management approaches have been recommended to farmers based on agro-ecological characteristics in different regions for aflatoxin management (Boshou et al. 2009). To increase breeding efficiency, studies on mechanisms of resistance to preharvest aflatoxin contamination were conducted and the most promising mechanisms identified were resistance to drought and root-knot nematode (Holbrook et al. 2009).

Screening for Stem and Pod Rot. Breeding lines along with known susceptible controls are screened in sick plots in replicated trials. Field screening under uniform, high disease pressure is a useful way to identify resistant genotypes for stem rot disease (Shokes et al. 1996; Pande et al. 1994), but this method has limitations like presence of natural antagonists and aggregation of inoculum resulting in disease escape. Low pathogen populations can also be a cause of concern in field screening. To overcome the limitations, it is desirable to use the same field each year to encourage build-up of inoculum in the soil. To facilitate pod rot development, it is important to increase the interval between irrigations during pod development stage. Shew et al. (1987) used oat seed inoculation method to increase the pathogen population and ensure uniform distribution in the sick plot. Shokes et al. (1998) followed a method of inoculating individual plants to improve the precision of screening. It was described as "agar disc technique" which is effective over field screening and oat inoculations. Breeding lines and cultivars were evaluated by inoculating 55–65 days old plants with aggressive isolates of *S. rolfsii* that were grown on grain-based (oats, corn) medium in the laboratory and the cultivars with maximum yield under disease pressure were selected and some of them were released in 2002 and 2003 (Gorbet et al. 2004).

Greenhouse screening, a simple and commonly used screening technique (Shew et al. 1987; Pande et al. 1994), for *S. rolfsii*, is described below:

1. Isolate *S. rolfsii* by hyphal-tip culture on potato dextrose agar
2. Prepare mycelial or sclerotial inoculum from young cultures of highly virulent isolates grown on dehydrated autoclaved groundnut shells or sorghum grains
3. Grow groundnut seedlings in 15-cm diameter pots containing a 1:2 mixture of sand and greenhouse potting mix
4. Add ten mature, well-dried sclerotia along with mycelial growth to the pots 8 weeks after sowing or just at pegging

5. Cover the surface with cloth and keep it wet to ensure soil surface and crown are kept humid
6. Incubate the inoculated plants in a green-house at 28–30 °C and relative humidity >85 %,with 12-h light and dark periods
7. Harvest the plants 30 days after inoculation and count the lesions on stems. Calculate average length of the three longest lesions on each stem

5.3.3.8 Virus Diseases

Groundnut is host to several virus diseases, but only a few of them are economically important - groundnut rosette disease (GRD) in Africa, peanut bud necrosis disease (PBND) in India, tomato spotted wilt virus (TSWV) in USA, peanut stripe potyvirus (PStV) in East and South East Asia, peanut stem necrosis disease (PSND) in pockets in Southern India and peanut clump virus disease (PCVD) in West Africa. In 1995, GRD epidemic affected approximately 43,000 ha of groundnut in Eastern Zambia with an estimated loss of US$4.89 million. In the following year, groundnut production in Malawi was reduced by 23 % due to GRD epidemic (Waliyar et al. 2007). The loss in pod yields vary with the strain type of peanut stripe poty virus (PStV) and it can reach as high as 55 % in China (Kunrong et al. 1999). PSND came to notice in India in 2000, when it caused an epidemic in Anantapur district in Andhra Pradesh affecting 225,000 ha and causing an economic loss of US$65 million (Reddy et al. 2002). Effective laboratory and field screening techniques have been developed to screen for resistance to these viruses. Sources of resistance were identified for GRD and PBND and used in breeding programs. Some wild diploid species have been identified as resistant to PStV.

Groundnut Rosette Disease (*GRD*). GRD is transmitted by *Aphis craccivora* and three agents are involved in causing the symptoms. They are groundnut rosette virus (GRV), groundnut rosette assistor virus (GRAV) and satellite RNA. An effective field screening method for GRV resistance is in operation in breeding programs in Africa (Nigam and Bock 1990). This method involves planting of infector rows of a susceptible variety after two rows of test genotypes, followed by transplanting infected plants that are heavily aphid infested at every 1.5 m among the infector rows after seedling emergence. Further, the infection is supplemented by releasing glasshouse-raised viruliferous aphids in the screening field. In order to identify and eliminate escapes from the apparently healthy plants in the field, the apparently healthy plants are individually harvested and their progenies are screened for GRV resistance in the glasshouse following mechanical sap inoculation. The test plants in glass house are inoculated with viruliferous aphids fed on GRAV infected plants or by grafting scion from GRAV-infected plants (Olorunju et al. 1992; Naidu and Kimmins 2007).

Olorunju et al. (1991) devised a method of estimating disease severity index (DSI) that was modified by Subrahmanyam et al. (1998) by reducing the individual plant disease scoring scale to a 1–3 scale, where 1 = plants with no visible disease symptoms on foliage and no stunting, 2 = plants with obvious rosette leaf symptoms

stunted to about 50 % of the size of symptom less plants and 3 = plants with severe rosette leaf symptoms and stunting greater than 50 %. Disease severity index (DSI) is calculated based on the score. Resistance to GRD was discovered in the late 1950s in local landraces of Burkina Faso. By utilizing them, cultivars resistant to GRD, such as K 11149A, K1124D, 69–101, RMP-91 and RG 1 were bred and released in Africa. These cultivars are now used as sources of resistance as the land races were semi-erect and late maturing (Bockelee-Morvan 1983; Mayeux et al. 2003) and resistant varieties with 19–92 % higher yield than susceptible were released in Malawi and Nigeria (Ntare et al. 2002).

Peanut Bud Necrosis Disease (*PBND*). The disease is caused by peanut bud necrosis virus (PBNV) and is transmitted by thrips, *T. palmi*. Screening for resistance to this disease is done in endemic areas with infector rows of susceptible plants (ex. Cowpea) sown to ensure sufficient inoculum load. In Thailand, Pensuk et al. (2002) found field disease incidence at 50 or 60 DAS as most appropriate parameter to identify resistance to PBNV in groundnut genotypes. Ten plants in each plot were randomly selected and disease score on a 1–5 scale for PBNV on each plant were recorded where 1 = healthy plant, 2 = spots on some leaves but no systemic symptoms, 3 = systemic symptoms without stunting, 4 = systemic symptoms with stunting and 5 = severe necrosis or die as described by Pensuk et al. (2002). The genotypes are then rated based on percentage of infected plants (Buiel 1995), the scoring of the infected plants is done every 2–3 weeks. Testing for PBNV resistance by mechanical inoculation under controlled Greenhouse conditions can also be used (Dwivedi et al. 1995).

Tomato Spotted Wilt Virus (*TSWV*). TSWV is transmitted by thrips in a persistent manner but it is not seed or pollen borne (German et al. 1992; Peters 2003). TSWV and related viruses have a wide host range and are reported to infect over 650 species of plants among both monocots and dicots (Culbreath et al. 2003). Field screening, similar to that used for PBND, can be adopted for TSWV screening. Culbreath et al. (1997) described a new intensity rating method based on percent of row length severely affected by TSWV, which takes much less time and effort than determining disease incidence based on individual plants and this is a practical alternative to individual plant assessment for characterization of genotype responses to TSWV. For stable resistance across locations, a multilocation field screening of genotypes is required due to potential strain variation in TSWV (Culbreath et al. 2000). A glasshouse screening method involving mechanical transmission protocol is also described for confirmation of field observations (Mandal et al. 2001).

Peanut Stripe Virus Disease (*PStVD*). Peanut stripe potyvirus (PStV) is transmitted by aphids, A. *craccivora*, *A. gossypii* and *Myzus persicae*. It is also seed-transmitted. Wongkaew and Dollet (1990) grouped isolates of PStV, obtained from different countries, into eight strains. Field screening for PStV under infector rows of a susceptible variety at regular interval is followed. Wakman and Ansar (1989) transplanted PStV infected plants in infector rows and also released aphids onto infected plants. Planting of the screening nursery at a time when natural aphid activity is more (dry season) will ensure better spread of the virus in the field. Scoring for PStV reaction is done based on percentage disease incidence, types of symptoms

observed, and yield estimation (Middleton et al. 1988). To improve the efficiency, screening has to be done in locations with high incidence of PStV.

Peanut Mottle Virus Disease (*PMVD*). Mottle disease, caused by peanut mottle potyvirus (PMV) is transmitted in a non-persistent manner by several aphid species including *Aphis craccivora* and infected groundnut seeds. Screening for resistance to PMV has been done under greenhouse conditions following mechanical sap inoculation and aphid transmission. The disease reaction is determined by symptoms.

Peanut Stem Necrosis Disease (*PSND*). It is caused by tobacco streak ilavirus (TSV) and transmitted by adults of thrips species, *F. schultzei*, *S. dorsalis* and *Megalurothrips usitatu*. A screening method, where *Parthenium* was grown one month before sowing the test genotypes around the field in which PSND screening would be carried out, gave encouraging results. An artificial inoculation method involving infected sap dilution at 1:10 and inoculation twice at 12 and 15 days after sowing was found to be very good in screening groundnut germplasm and to identify stable resistance (Nigam et al. 2012). Screening for TSV/PSND resistance should be carried out when temperature conditions are favorable (28–32 °C) for virus multiplication and symptom expression.

Peanut Clump Virus Disease (*PCVD*). It is caused by a peanut clump furovirus (PCV) and is transmitted by soil inhabiting fungus *Polymyxa graminis*. Hot spot locations have been used for screening for resistance to peanut clump disease. A convenient and reliable glasshouse screening method was suggested by Reddy et al. (2005) using mechanical sap inoculation, where French bean is used as source of inoculum.

5.3.3.9 Bacterial Diseases

Bacterial wilt is most predominant among bacterial diseases of groundnut. It is caused by *Ralstonia solanacearum.* It was first reported from Indonesia (1905) and later in Georgia, USA (1931). Presently, the disease is one of the major biotic constraint in China, Indonesia and Vietnam. Yield losses range from 10 to 30 %. In China, annual losses in groundnut pod yield due to bacterial wilt are estimated over 50,000 t (Mehan et al. 1994). Evaluation of breeding lines for wilt resistance is largely based on field screening in wilt-sick plots under uniform high disease pressure. Screening in hot-spot locations of China, Vietnam and Indonesia is common. Greenhouse screening using pure culture, controlled soil temperature and moisture and inoculum concentration and placement can give more precise information. Several sources of resistance originating from Indonesia and China are used in breeding programs in groundnut growing countries in East and South East Asia. For field screening, test genotypes are sown in replicated plots along with susceptible checks, arranged systematically throughout the wilt-sick filed (Sharma and Soekarno 1992) and percentage of wilted plants in each genotype are recorded based on visual observations. Lines showing up to 10 % wilt incidence are considered highly resistant and those with 10–20 % incidence are resistant. Lines with less than 30 % survival are highly susceptible (Mehan et al. 1994). Following extensive screening of

about 5,000 breeding lines and germplasm accessions in wilt-sick plots in China and Indonesia, many lines with varying levels of resistance have been reported (Duan et al. 1993; Sharma and Soekarno 1992; Mehan et al. 1994). Several glasshouse screening techniques resulting in successful inoculation with pure cultures of bacterium have been developed using plants at seedling stage. These include stem inoculation (stem puncture), hypodermic injection and root inoculation (Kelman 1953). Of which, root inoculation technique appears to be the best way to evaluate the plants for resistance, while stem inoculation may eliminate certain lines which might have field resistance (Mehan et al. 1994). Soaking seeds in bacterial suspension (6×10^8 cfu ml^{-1}) for 30 min is another useful inoculation technique (Li and Tan 1984). Infested soil placed in the pots or other containers can also be used as a source of inoculum for screening under controlled conditions.

5.3.3.10 Nematodes

Globally, nematodes cause 11.8 % of pod yield losses in groundnut. The root-knot nematodes, *Meloidogyne* spp. and the lesion nematodes, *Pratylenchus* spp. are important in groundnut (Sharma and McDonald 1990). The root-knot nematode causes substantial yield losses in severely infested fields, resulting primarily from stunted plant growth and premature plant death. The parasitic species, *M. areneria*, *M. javanica* and *M. hapla* have worldwide distribution, while *M. incognita* was not found to be parasitic so far on groundnut (Sharma and McDonald 1990). Only race 1 of *M. areneria* and *M. hapla* is parasitic in USA, India and China, while *M. javanica*, common in Egypt and India is not parasitic in USA. Kalahasti malady, a nematode disease caused by *Tylenchorhynchus brevilineatus* causes brownish-black discoloration on pod surface and reduced pod size was first observed in 1975–1976 in Chitoor district of Andhra Pradesh, India. Since then the disease has been widespread and serious.

Screening for Nematode Resistance. Resistance of plant-parasitic nematodes is commonly defined as a reduction or inhibition of nematode reproduction. Phenotyping can be done following the screening procedure described by Holbrook et al. (1983) for resistance to *M. arenaria*. In this method, plants were inoculated with 3,500 eggs of nematode prepared using the NaOCl method (Hussey and Barker 1973) and applied 10 days after planting. Approximately 70 days after inoculation, the roots were placed in 1,000 ml cups containing 300 ml of 0.05 % (v/v) phloxin B solution for 3–5 min. Each plant was indexed for root galls and egg masses based on a scale of 0–5 (0 = no galls or no egg masses, 1 = 1–2, 2 = 3–10, 3 = 11–30, 4 = 31–100, and 5 = more than 100 galls or egg masses per root system). To identify resistant source for *Tylenchorhynchus brevilineatus* (Kalahasti malady disease), screening in farmer's field in Chittoor district of Andhra Pradesh, a hot spot location was followed. The nematode density was estimated using a modified Baermann funnel technique (Southey 1970). The disease scoring was done on a 1–5 scale in which 1 = no disease symptoms evident; 2 = a few small dark brown to black lesions to cover 1–25 % on some pods, pods of normal size; 3 = many small lesions coalescing

to cover 25–50 % of pod surfaces, all pods affected, pods of normal size; 4 = many lesions coalescing to cover 50–75 % of pod surfaces, all pods affected, pods of smaller than normal sixe; and 5 = many lesions coalescing to cover 75 % of pod surfaces, all pods affected, pods of much smaller than normal size (Mehan et al. 1993). The screening methods were useful to identify resistant source and breed cultivars with resistance to root knot nematodes (Simpson et al. 2003). A tolerant cultivar to Kalahasti malady, Tirupati 3 has been released for cultivation in endemic areas in India (Mehan et al. 1993).

5.3.3.11 Insect Pests

Aphids (*Aphis craccivora* Koch), three different species of thrips (*Frankliniella schultzei*, *Thrips palmi* and *F. fusca*), leaf miner (*Aproaerema modicella*), jassids (*Empoasca kerr*i and *E. fabae*) and *Spodoptera* are the major pests in groundnut, among which aphids, thrips and *Spodoptera* have worldwide distribution and cause serious damage (Whitman and Amin 1988). In addition, termites, white grubs and storage pests also cause damage to the groundnuts. Detection of host–plant resistance to insect pests is a lengthy procedure and has to be carried out with maximum care. Plant resistance to major insect pest in cultivated and wild species of *Arachis* has been confirmed to the following species: thrips, aphids, leafhoppers, *Helicoverpa* sp., *Spodoptera* sp., and leaf miner (Lynch 1990). However, tapping of resistance to insect pests from wild species into cultivated species has not been successful so far (Sharma et al. 2003). Screening procedures for resistance to common insect pests are described in detail by Ranga Rao and Wightman (1996). The technique employed for screening differs with insect involved and sometimes location itself.

Screening for Resistance to Aphids. Resistance to aphids is important as they transmit major virus diseases. Field screening of breeding lines is done along with known susceptible checks under heavy infestation [>100 aphids per plant at 30 days after emergence (DAE)] under natural conditions, where ten plants are selected and total number of aphids on them are recorded (Padagham et al. 1990). This procedure is tedious, thus for mass screening the lines with ≤50 % infestation than the susceptible control are selected and further evaluated for confirmation by screening in glasshouse. Glasshouse screening is done by transferring two adult aphids on each plant (15–20 DAE) using a fine tipped camel hair brush and scoring is done based on number of aphids developed on each plant. At least ten single plants should be taken for each genotype for the glasshouse screening and lines with 50 % less infestation than the susceptible control are selected (Zeyong et al. 1995).

Screening for Resistance to Thrips. Resistance to thrips is important as they also transmit major virus diseases. Field screening is done by sowing the test genotypes along with highly susceptible lines or infector rows of a susceptible crop such as cowpea to coincide with peak periods of thrips infestation/migration followed by scoring for the thrips damage (Amin et al. 1985). The screening should coincide with period of peak infestation/migration, which varies with location and season at

a given location. Ekvised et al. (2006) suggested that plant damage parameters are more useful than thrips number in identifying differences among groundnut cultivar as these parameters are more consistent across evaluation dates and years. A rating scale of 1–9 for scoring thrips injury is used, where 1 = 0–10 % damage, 2–3 = 11–30 % damage, 4–5 = 31–50 % damage, 6–7 = 51–70 % damage and 8–9 = 71–100 % damage (that is also read as 1 = highly resistant, 2–3 = resistant, 4–5 = moderately resistant, 6–7 = susceptible, and 8–9 = highly susceptible (Ranga Rao and Wightman 1996; Dwivedi et al. 1995).

Screening for Resistance to Spodoptera. Since this pest is highly sporadic on farm, a simple effective artificial filed screening technique was developed at ICRISAT (Ranga Rao and Wightman 1996). In this method, test genotypes are sown along with known susceptible checks in replicated design and the area is surrounded by 15 cm aluminum barrier to arrest the escape of the larvae from the experimental area. Another set of test material should be planted outside the barrier to have a pest-free comparison. The artificially reared fourth instar larvae from the insectary are released in test rows planted in the field. Ten random plants from the central rows are selected and leaf area is measured and relative performance of lines is assessed based on loss of leaf area. The genotypes with less than 20 % damage were identified as resistant (Ranga Rao and Wightman 1996).

Screening for Resistance to Leaf Miner. Field screening is done by growing test entries along with known susceptible controls in replicated design. However, sporadic nature of the pest makes field screening non-reliable over the years of testing. At ICRISAT, an artificial screening method under laboratory conditions was developed (Ranga Rao and Wightman 1996). This involves maintaining leaf miner cultures in small cages under glasshouse and after obtaining moths from the insectary, 30 pairs of moths per cage are released on test entries. Resistance to leaf miner is assessed by following 1–9 scale in 20 leaves collected at random (Ranga Rao and Wightman 1996). The lines having less than 20 % damage are classified as resistant.

Screening for Tolerance to Jassids. Screening for resistance to jassids is done under field conditions by growing test entries along with known susceptible controls in replicated design. The screening nursery is preferably grown to coincide the natural peak infestation of jassids (Ranga Rao and Wightman 1996). At ICRISAT center the peak infestation is seen during August-September and February–March. During peak population periods scoring should be done for jassid injury on a scale of 1–9. The scoring has to be done at least twice with 15 days interval. Resistance can also be estimated by counting the percentage of yellowed foliage by visual rating at time of peak infestation from 10 leaves randomly collected from 3, 4 or 5 leaf positions on the main stem, in a plot of 12.5 m^2 (Dwivedi et al. 1986).

Storage Pests. Groundnut borer or weevil or bruchids (*Caryedon serratus*) and rust-red flour beetle (*Tribolium castaneum*) are major storage pests in groundnut. Others include merchant grain beetle (*Oryzaephilus Mercator*), Khapra beetle (*Trogoderma granarium*), *Elasmolomus sordidus*, and rice moth (*Corcyra cepahlonica*). Groundnut borer, found in Asia and Africa, is the only species that can penetrate

intact pods to infest the kernels. Rust-red flour beetle is distributed throughout the tropics and is major pest on shelled groundnuts. Breeding for resistance to storage pests has not been an objective in groundnut breeding programs, nevertheless, evaluation of advanced breeding lines for plant resistance to post-harvest infection of storage pests is important, as the new high-yielding varieties have frequently proved to be more susceptible to insect attack during storage than the indigenous genotypes (Dick 1987). The screening should be performed under controlled temperature and humidity as they influence the length of the insects' development period. Known susceptible and resistant genotypes should be included in the screening and the duration of the storage in the experiment should be designed as per the requirement. The parameters such as loss of pod/kernel mass, length of development period of the pest, mortality of juvenile stages, amount of food consumed and oviposition rate of the storage pest can be assessed to indicate the cultivar resistance to storage pest infections (Dick 1987). The differences in these parameters obtained in screening trials reflect differences between genotypes when both the kernel and insects used in the experiment are uniform. The insect cultures to supply insects for screening should be relatively constant in density and the kernel should be preconditioned to the experimental temperature and humidity for a period of at least 2 weeks.

5.3.4 Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) in groundnut can be improved through both, cultivar selection and *Rhizobium* strain improvement (Nambiar et al. 1982). In breeding programs the genotypes with high BNF can be selected based on various parameters such as, nodule number, nodule mass, top weight and total nitrogen (Wynne et al. 1980; Nambiar et al. 1982) and nitrogenase activity (Nigam et al. 1985). Of these parameters, nodule number and nodule mass and top weight is simple to measure and most commonly used. Nitrogenase activity is measured using acetylene reduction (Ar) assay (Herdina and Silsbury 1990), carried out in a closed vessel containing 10 % acetylene using detached nodules, de-topped roots, or whole plants. Gas chromatograph (GC) is used to determine the amount of ethylene formed and expressed as nano-moles or micromoles of ethylene produced per hour per plant or per weight unit of nodules. The acetylene reduction assay provides a measure of nitrogenase activity under the experimental conditions and it can vary on field based on seasonal conditions and moreover it does not measure atmospheric nitrogen that is fixed by the plants hence not frequently used. Total leaf nitrogen is another parameter to indicate BNF of genotypes and it is determined by Kjeldhal method, discussed in detail under seed protein content estimation. It can also be estimated by robust methods such as, Technicon Autoanalyser (Pulse Instrumentation Ltd, Saskatoon, SK) (Singh and Jambunathan 1980) or near infrared reflectance spectroscopy (NIRS) (Misra et al. 2000). At ICRISAT (ICRISAT Annual reports, 1981) and North Carolina State University, Raleigh, USA (Wynne et al. 1980, 1983) high performing germplasm lines were identified based on evaluation of above parameters.

5.3.5 *Confectionary and Nutritional Traits*

Traits for confectionary purposes are important for both, food uses and export markets. For confectionary uses, groundnuts are bred possessing all/some of these traits: greater proportion of sound mature kernels (SMK), flavor, 100 seed weight exceeding 55 g, >11 % of sugar content, >24 % of protein content, blanchability (>60 %) and low oil content (<45 %) (Ramanathan 2004) and variability for these traits is already known (Dwivedi and Nigam 2005). Seed coat colour and seed shape are the other important confectionary attributes. There are several nutritional attributes, for which groundnut improvement is targeted, of which protein and oil content and fatty acids composition are important. While low oil content is preferred for confectionary uses, it is high oil content that is important for oil extraction as high oil content in groundnut is translated into economic benefits to both farmer and millers (Narasimham et al. 1985). It is known that fatty acid composition determines oil quality; oleic and linoleic acids account for 80 % of the fatty acids found in groundnut oil. Groundnuts are bred for high oleic to linoleic ratio. Gorbet and Knauft (1997) registered the first high oleic line, SunOleic 95R, and more cultivars were developed since then (Chu et al. 2011).

Phenotyping for both, confectionary and nutritional traits involves analysis of groundnut kernels, therefore they are discussed together under physical and chemical traits.

5.3.5.1 Physical Traits

Sound Mature Kernels (SMK) and Seed Size, Shape and Color. Higher proportion of sound mature kernels (SMK) is an important attribute as it indicates proportion of fully mature kernels. SMK % is the ratio of weight of SMK to weight of total kernels (that includes immature/shriveled kernels). Depending upon the end use seed size also become an important consideration in confectionery groundnuts. Seed size is measured in counts (number of seeds per ounce in trade) or as seed length (mm) and seed width (mm), and 100-seed weight (g) Dwivedi and Nigam (1995). The US peanut kernel grades based seed count are as follows: Virginia Extra Large—28/32 counts/oz, Virginia Medium—38–42 counts/oz, Virginia # 1—45/55 counts/oz, Runner Jumbo—38/42 counts/oz, Runner Medium—40/50 counts/oz, Runner # 1—60/70 counts/oz, Spanish Jumbo—60/70 counts/oz, Spanish # 1—70/80 counts/oz. Kernels are also graded using grading sieves with holes of prescribed dimensions (NPCA 1988). The seed measurements also reflect the shape of the seed. When used as roasted-in-shell, pod traits–pod size, pod shape, pod appearance and cleanliness etc become important. Groundnut testa color varies from light brown to deep red and 20 different testa colors are known, of which the preferred colours are tan, rose tan and red (Dwivedi and Nigam 2005). Red testa colour is a preferred trait in snack industry. Seed coat colour is scored based on visual observations taking care to avoid recording observations on stored seed as seed coat upon storage turns darker.

Blanchability. Blanchability is removal of testa or seed coat (skin) from raw or roasted groundnuts and this attribute is of economic importance in processed groundnut food products, which include peanut butter, salted groundnuts, candies, and bakery products and groundnut flour. To determine the blanchability of genotypes a laboratory type blancher, based on the model developed by Wright and Mozingo (1975), was fabricated at ICRISAT Center (Singh et al. 1996). A preheating temperature of 110 °C for 35 min, with 200 g sample for blanching time of 2 min (120 s) and blanching pressure of 17.6 psi was standardized for blanching at ICRISAT. The following blanchability parameters are taken into account when breeding lines are selected for this trait—total blanchability (TB, includes fully blanched intact kernels and fully blanched splits), whole blanched (WB, fully blanched intact kernels), whole unblanched (UB, unblanched intact kernels), partially blanched (PB, partially blanched intact kernels), blanched splits (SB, fully blanched splits) and unblanched splits (UBS).

5.3.5.2 Chemical Traits

Chemical analyses can be done on random samples or samples consisting of only SMKs. If needed the samples may be divided into sub-samples and the mean of the sub-sample readings can be taken to improve the accuracy of estimation.

Flavour and Sugars. The sensory attributes that make up roasted peanut and flavor quality are important traits to evaluate in development of new cultivars. Roasted groundnuts are evaluated by organoleptic test by a carefully selected "taste panel" for flavor attributes. The desirable flavors include almond, coffee, fresh, nutty, popcorn, smoky and sweet and not off-flavour (Fletcher 1987). Firm and crispy texture of the roasted groundnuts is preferred and soft and mushy roasted groundnuts lack consumer preference. Free sugars and amino acids have been found to be the major flavor precursors in roasted groundnut (Newell et al. 1967). Seed sugars provide a source of carbon for the production of flavour compounds and also impart desirable taste. Soluble sugars of raw groundnuts are estimated by extracting sugars from defatted flour from freeze dried and cold stored groundnuts samples using 80 % methanol and fractured by high performance liquid chromatography (HPLC) (Basha 1992). A simpler colorimetric method for sugar determination uses phenol–sulfuric acid (Dubois et al. 1956). Sugar can also be estimated by Anthrone reagent method in which carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm. Total sugars are expressed as per cent of total seed weight.

Seed Oil Content and Fatty Acids. Oil content is estimated by Soxhlet method, a gravimetric approach that involves estimation of solvent extracted oil from a given quantity of ground sample. More robust methods like nuclear magnetic resonance (NMR) (Jambunathan et al. 1985) and near infrared reflectance spectroscopy (NIRS)

(Misra et al. 2000) are also used. A high correlation (r=0.97) between the estimates of Soxhlet and NMR methods was reported by Jambunathan et al. (1985). For NMR oven dried samples are used to determine oil content. NIRS facilitates non-destructive method of estimation and single-intact kernel (Fox and Cruickshank 2005) or pod (Sundaram et al. 2010) can be used for estimating oil content and fatty acids. It can also be done using grounded meal sample. Single-seed based oil content determination enables screening of segregating populations and reject the low oil content seeds at early generations thus optimize both, time and resources. The oil content determined by Soxhlet method is used to both, calibrate and validate NIRS. The fatty acid analysis of breeding lines is carried out on a gas chromatograph (GC) by estimating fatty acid methyl esters (Phillips and Singleton 1981). NIRS can be used for robust estimation of fatty acid, but prior calibration and validation with readings of GC is required. Oil and fatty acids are expressed as per cent of seed weight.

Seed Protein Content. The wet chemistry method, Kjeldhal procedure is used for estimation of nitrogen content which can then be converted to protein content. Quantifying nitrogen content by Kjeldhal method involves digesting the sample in strong acid such as, sulphuric acid to produce ammonium sulphate, followed by liberation of ammonia by adding strong alkali (sodium hydroxide). The ammonia is then captured by boric acid and the exact amount of nitrogen is determined by titrating the excess acid with sodium carbonate. The nitrogen content thus estimated is expressed as protein content after conversion, the conversion factor for groundnut is 5.46. Although Kjeldhal method is fairly accurate, it is quite cumbersome and time-consuming and hence robust methods of determining protein content such as, Technicon Autoanalyser (Pulse Instrumentation Ltd, Saskatoon, SK) (Singh and Jambunathan 1980) or NIRS (Misra et al. 2000) can also be used. Protein content of seed is expressed as per cent of seed weight.

Estimation of Iron and Zinc Content and Other Nutritional Factors. Triacid method was used for digesting the groundnut seed samples and then Fe and Zn contents are measured by atomic absorption spectrometer (AAS). For which one gram ground sample is digested with 10 ml triacid mixture consisting of nitric acid, sulfuric acid and perchloric acid in the ratio of 10:0.5:2 (v/v). Digestion is done overnight (for cold digestion) in digestion chamber. The sample is digested initially at 120 °C for 1 h followed by digestion at 230 °C for about 2 h to get clear and colorless solution. The digestion tubes were allowed to cool down and the contents were dissolved in water and diluted to 75 ml with distilled water. This aliquot is taken for the estimation of Fe and Zn concentration. The concentrations are measured by AAS, Varian Spectra AA 20 and results were expressed in mg kg^{-1} (Sahrawat et al. 2002). Same procedure is used for estimation of other micronutrients like calcium, potassium, magnesium, manganese and copper. X-ray diffraction spectroscopy (XRF), a non-destructive method that does not require digestion of the samples can be more useful when large number of breeding populations and genotypes are to be studied. The other nutritional factors such as, niacin (Whitley et al. 2011), tocopherols, folic acid (Dean et al. 2009), proanthocyanidins (flavonoid), and quercetin (flavonols) (Choo and Siong 1996; Wang et al. 2008) were quantified in groundnuts using high performance liquid chromatography (HPLC).

5.3.6 Haulm Yield and Quality

Groundnut haulms fodder is used for livestock and it is this important dual purpose usage of groundnut that prompted groundnut breeders and livestock nutritionists to collaboratively explore the feasibility of genetic enhancement of not only pod traits but also haulm yield and haulm quality. No inverse relationships exist between haulm fodder quality traits and pod and haulm yield, which is important to improve the haulm fodder quality without jeopardizing the pod yield (Nigam and Blummel 2010). Harvested groundnuts are air dried in the field, after which the pods were stripped and biomass weighed to determine haulm yield (kg ha^{-1}). Although haulm yield has been an important parameter in selecting genotypes, determining haulm quality of the breeding lines is not used often. Haulm nitrogen content, in vitro organic matter digestibility (OMD) (%) and metabolisable energy (ME) (MJ kg^{-1}) are important parameters for which breeding line are evaluated to determine haulm quality. Haulm quality is analyzed for haulm nitrogen content done by Kjeldhal method as described above. Estimation of OMD and ME are described by Menke and Steingass (1988). About 200 mg samples were placed in polyester/polyethylene bags (size 5 cm×3 cm; pore size 25 μm), incubated at 39 °C with 35 ml rumen liquor-buffer mixture in 100 ml glass syringes and measured after 0, 3, 6, 12, 24, 48, 72 and 96 h incubation. After finishing the in vitro digestion trials, bags were gently rinsed with cold tap water and dried at 65 °C for 48 h to determine OMD. The residues were analyzed for Organic Matter (OM) and Organic Matter Digestibility (OMD). Each measurement was performed in triplicate. Gross Energy (GE) content was determined by PARR6300 (ARC 1965) and ME is determined by equation, ME = GE × OMD × 0.815. Near infrared reflectance spectroscopy (NIRS) can be used to determine haulm quality parameters. The NIRS is calibrated for haulm nitrogen content, OMD and ME based on wet chemistry readings and then used for determining haulm nitrogen content, OMD and ME of haulm (Nigam and Blummel 2010). After recording weight the haulms were ground to pass through a 1 mm sieve particle mesh and such a fine powder is used for haulm quality analysis. The ground samples are scanned on NIRS to determine haulm quality traits.

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